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Abstract: The MutL family of mismatch repair proteins belongs to the GHKL class of ATPases, which contains also type II topoisomerases, HSP90, and histidine kinases. The nucleotide binding domains of these polypeptides are highly conserved, but this similarity has failed to help us understand the biological role of the ATPase activity of the MutL proteins in mismatch repair. hMutLalpha is a heterodimer of the human MutL homologues hMLH1 and hPMS2, and we decided to exploit its asymmetry to study this function. We now show that although the two subunits contribute differently to the ATPase activity of the heterodimer, hMutLalpha variants in which one subunit was able to bind but not hydrolyze ATP displayed similarly reduced mismatch repair activities in vitro. In contrast, variants in which either subunit was unable to bind the nucleotide were inactive. Mutation of the catalytic sites of both subunits abolished repair without altering the ability of these peptides to interact with one another. Since the binding of the nucleotide in hMutLalpha was not required for the formation of ternary complexes with the mismatch recognition factor hMutSalpha bound to a heteroduplex substrate, we propose that the ATPase activity of hMutLalpha is required downstream from this process.

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Mutations within the hMLH1 and hPMS2 Subunits of the Human MutLα Mismatch Repair Factor Affect its ATPase Activity, but not its Ability to Interact with hMutSα.

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ABSTRACT

The MutL family of mismatch repair proteins belongs to the GHKL class of ATPases, which contains also type II topoisomerases, HSP90 and histidine kinases. The nucleotide binding domains of these polypeptides are highly conserved, but this similarity has failed to help us understand the biological role of the ATPase activity of the MutL proteins in mismatch repair. hMutLα is a heterodimer of the human MutL homologues hMLH1 and hPMS2 and we decided to exploit its asymmetry to study this function. We now show that although the two subunits contribute differently to the ATPase activity of the heterodimer, hMutLα variants in which one subunit was able to bind – but not hydrolyze – ATP, displayed similarly reduced mismatch repair activities *in vitro*. In contrast, variants in which either subunit was unable to bind the nucleotide were inactive. Mutation of the catalytic sites of both subunits abolished repair without altering the ability of these peptides to interact with one another. As the binding of the nucleotide in hMutLα was not required for the formation of ternary complexes with the mismatch recognition factor hMutSα bound to a heteroduplex substrate, we propose that the ATPase activity of hMutLα is required downstream from this process.

INTRODUCTION

DNA mismatch repair (MMR) is a highly conserved process that removes nucleotides misincorporated into the newly-synthesized DNA strand during replication or recombination (1,2). Unlike other DNA repair pathways, correction of biosynthetic errors requires not only that non-canonically paired bases be recognized, but also that the repair system be able to differentiate between the template and the newly-synthesized DNA strand, such that it can direct the correction process to the latter. In *E.coli*, mismatched base pairs and short insertion-/deletion loops (IDL) are recognized by the MutS homodimer, which initiates the repair process by recruiting the remaining members of the repairosome: the MutL homodimer, the strand discrimination factor MutH and DNA helicase II (UvrD), as well as one of several exonucleases (both 5'→3 and 3→5), DNA polymerase III holoenzyme and DNA ligase. The MutL protein plays a key role in coordinating this process. It activates the cryptic endonuclease activity of MutH (3-5), which then nicks the newly-synthesized DNA strand. This reaction is enabled by the transient undermethylation of GATC sequences in newly-synthesized DNA and by the ability of MutH to incise only the unmethylated strand. MutL also appears to be responsible for directional loading of DNA helicase II at the site of the nick (6,7). The displaced DNA is exonucleolytically degraded and the removed stretch is then resynthesized by the replicative DNA polymerase (see (8,9) for review). In spite of the fact that the MMR pathway of *E.coli* could be reconstituted from the purified individual components (10), our knowledge of the molecular mechanism of the process is limited. This is especially true for the MutL protein, the role of which is particularly enigmatic. What is clear is that its transactions, like those of MutS, are governed by ATP binding and/or hydrolysis. This was first implied by experiments of Grilley et al. (11), who showed that MutL and MutS interact on a heteroduplex substrate in an ATP-dependent manner, and from i*n vivo* studies of Aronshtam and Marinus (12), who showed that MutL alleles carrying single amino acid substitutions in the vicinity of the ATP binding domain of MutL were unable to complement the MMR defect of a *mutL*$^{-}$ strain. More recently, the N-terminal domain of

MutL could be shown to contain four short sequence motifs that are found also in the type II topoisomerases, the HSP90 heat shock proteins and histidine kinases (Fig. 1a) (13,14). Structural analyses of members of all four families revealed that these proteins share a novel nucleotide-binding fold and suggested an important role for the invariant residues in binding and/or hydrolysis of ATP (see (15) for review). Thus, this superfamily of proteins, known as GHKL ATPases, is likely to have evolved from a common ancestor. However, as illustrated by the divergent roles of these proteins, their common fold is a poor predictor of biological function.

The recently-described crystal structures of LN40, the N-terminal 349 amino acid residues of MutL (16,17), revealed that the polypeptide folds into two α/β domains (Fig. 1a). The first (amino acids 20-200) harbors all four conserved motifs that form the nucleotide binding site. In the second domain (amino acids 224-331), a loop containing an invariant lysine residue (K307), is likely to act as a sensor that triggers a conformational change in the protein in response to nucleotide binding within the first domain. Such changes have been noted with both LN40 and the entire MutL protein (16). Analysis of gel filtration and sedimentation properties of the full length MutL suggested that its elongated structure became globular upon binding of ADPNP. More dramatically, binding of ADPNP triggered dimerization of LN40 in solution, as well as in crystallization experiments. The structure of LN40•ADPNP revealed that more than 60 amino acid residues within this domain became ordered and contributed to nucleotide binding or to the dimerization of the NBDs. Assuming that the carboxy-terminal ends of MutL remain stably associated during this time, the amino terminal part of MutL could function as an ATP-driven molecular gate. Type II topoisomerases make use of such a gate to capture a segment of duplex DNA in order to pass it through a transient double strand break (18) and ATP-induced dimerization of HSP90 (19) has been suggested to play a role in clamping the substrate polypeptides during the folding reaction (20). In addition, ATP binding/hydrolysis is important in the coordination of HSP90 interactions with other proteins. For example, HSP90 interacts with the co-chaperone p23 only in its ATP-bound form (21,22). Given

that MutL needs to interact with different partners, a similar role of ATP binding might be anticipated. Based on the observation that non-hydrolyzable ATP analogues promoted the binding of single-stranded DNA by MutL (17,23), it was hypothesized that the molecular gate might be used to clamp MutL onto DNA. In agreement with this, single stranded DNA was found to stimulate the very weak ATPase activity of MutL (17,24). Importantly, substitution of an arginine residue located within the gate (R266E) abolished both DNA binding and the stimulation of the ATPase activity (17).

The molecular mechanism of MMR appears to be highly conserved, as witnessed by the identification of MutS and MutL (but not MutH) homologues in most organisms studied to date. In human cells, the MutS homologues hMSH2, hMSH3 and hMSH6 form two heterodimers, hMutSα (hMSH2/hMSH6) and hMutSβ (hMSH2/hMSH3), which are involved in mismatch recognition (25-28). Of the four *mutL* homologues (*hMLH1, hPMS1, hPMS2* and *hMLH3)* identified to date in the human genome (29), *hMLH1* and *hPMS2* play a predominant role in MMR (30-35). The polypeptides encoded by these genes interact *via* their carboxy-terminal halves to form a stable heterodimer, hMutLα, which is indispensable for MMR (30,36-38).

Germline mutations in MMR genes, such as those found in the Hereditary Non-Polyposis Colon Cancer (HNPCC) kindreds, predispose to cancers of the colon, endometrium and ovary (39). Of the nearly 300 families entered in the HNPCC database (http:/www.nfdht.nl), more than 160 carry mutations in the *hMLH1* locus, of which about 30 % cause single amino acid changes. Many of these missense mutations are found in the highly conserved amino-terminal region, suggesting that they might affect the ATPase function of the hMLH1 protein (Fig. 1b). Interestingly, no corresponding mutations have been identified in the *hPMS2* gene, with only four families with germline mutations in this locus having been described to date (33,40,41). This implies either that the *hPMS2* gene is less prone to mutagenesis than the other HNPCC loci, that the function of hPMS2 is not essential for MMR due to a possible functional redundancy with another member of the MutL

homologue family, such as for example hMLH3 (42), or that missense mutations in hPMS2 escape detection, because they are phenotypically silent. We set out to address the latter hypothesis by studying the relative importance of the ATP binding domains of the two subunits of hMutLα in the MMR process. We could show previously that recombinant hMutLα, purified from baculovirus-infected insect cells, was able to complement *in vitro* the MMR defect of human cell lines carrying mutations in either the *hMLH1* or *hPMS2* genes (37). In the present study, we made use of this system by introducing defined amino acid substitutions into the ATPase active sites of hMLH1 and hPMS2 and by investigating the properties of the recombinant variants in several biochemical assays.

EXPERIMENTAL PROCEDURES

Site-Directed Mutagenesis and Production of Recombinant Baculoviruses- The Bac-To-Bac baculovirus expression system (Invitrogen) was used according to the instructions of the manufacturer. Vectors for the expression of the wild type proteins have been described in (37). Site-directed mutagenesis was used to generate vectors encoding MutL homologues that carry single amino substitutions in the ATP binding domain. *pFastBacI-hMLH1[E34A]:* Two PCR fragments amplified from *pFastBacI-hMLH1* using the primer pairs mr_34/mr_19 and mr_18/mr_2 were used as templates in a second PCR step, using the mr_34/mr_2 primers. The final PCR product was cloned between the *Bam*HI/*Pvu*II sites of *pFastBacI-hMLH1. pFastBacI-His$_6$-hMLH1[E34A]:* Two PCR fragments amplified from *pFastBacI-His$_6$-hMLH1* using the primer pairs mr_4/mr_19 and mr_18/mr_2 were used as templates in a second PCR step, using the mr_4/mr_2 primers. The final PCR product was cloned between the *Bam*HI/*Pvu*II sites of *pFastBacI-His$_6$-hMLH1. pFastBacI-hMLH1[N38A]:* Two PCR fragments amplified from *pFastBacI-hMLH1* using the primer pairs mr_34/mr_21 and mr_20/mr_2 were used as templates in a second PCR step, using the mr_34/mr_2 primers. The final PCR product was cloned between the *Bam*HI/*Pvu*II sites of *pFastBacI-hMLH1. pFastBacI-His$_6$-hMLH1[N38A]:* Two PCR fragments amplified from *pFastBacI-His$_6$-hMLH1* using the primer pairs mr_4/mr_21 and mr_20/mr_2 were used as templates in a second PCR step, using the mr_4/mr_2 primers. The final PCR product was cloned between the *Bam*HI/*Pvu*II sites of *pFastBacI-His$_6$-hMLH1. pFastBacI-hMLH1[D63N]:* Two PCR fragments amplified from *pFastBacI-hMLH1* using the primer pairs mr_34/mr_39 and mr_38/mr_2 were used as templates in a second PCR step, using the mr_34/mr_2 primers. The final PCR product was cloned between the *Bam*HI/*Pvu*II sites of *pFastBacI-His$_6$-hMLH1. pFastBacI-His$_6$-hMLH1[D63N]:* Two PCR fragments amplified from *pFastBacI-His$_6$-hMLH1* using the primer pairs mr_4/mr_39 and

mr_38/mr_2 were used as templates in a second PCR step, using the mr_4/mr_2 primers. The final PCR product was cloned between the *Bam*HI/*Pvu*II sites of *pFastBacI-His$_6$-hMLH1*. *pFastBacI-His$_6$-hPMS2:* A fragment containing the 5' end of the hPMS2 cDNA was amplified by PCR from *pFastBacI-hPMS2* using primer *mr55* and primer *mr41* and cleaved with *Bam*HI/*Afl*II. The resulting fragment was cloned between the *Bam* HI and *Afl*II sites of *pFastBacI-hPMS2*. *pFastBacI-hPMS2[E41A]:* Two PCR fragments amplified from *pFastBacI-hPMS2* using the primer pairs mr_34/mr_23 and mr_22/mr_26 were used as templates in a second PCR step, using the mr_34/mr_26 primers. The final PCR product was cloned between the *Bam*HI/*Afl*II sites of *pFastBacI-hPMS2*. *pFastBacI-His$_6$-hPMS2[E41A]:* Two PCR fragments amplified from *pFastBacI-His$_6$-hPMS2* using the primer pairs mr_34/mr_23 and mr_22/mr_26 were used as templates in a second PCR step, using the mr_34/mr_26 primers. The final PCR product was cloned between the *Bam*HI/*Afl*II sites of *pFastBacI-hPMS2*. *pFastBacI-hPMS2[N45A](#139):* Two PCR fragments amplified from *pFastBacI-hPMS2* using the primer pairs mr_34/mr_25 and mr_24/mr_26 were used as templates in a second PCR step, using the mr_34/mr_26 primers. The final PCR product was cloned between the *Bam*HI/*Afl*II sites of *pFastBacI-hPMS2*. *pFastBacI- His$_6$-hPMS2[N45A]:* Two PCR fragments amplified from *pFastBacI-His$_6$-hPMS2* using the primer pairs mr_34/mr_25 and mr_24/mr_26 were used as templates in a second PCR step, using the mr_34/mr_26 primers. The final PCR product was cloned between the *Bam*HI/*Afl*II sites of *pFastBacI-hPMS2*. *pFastBacI-hPMS2[D70N]:* Two PCR fragments amplified from *pFastBacI-hPMS2* using the primer pairs mr_34/mr_41 and mr_40/mr_42 were used as templates in a second PCR step, using the mr_34/mr_42 primers. The final PCR product was cloned between the *Bam*HI/*Pvu*II sites of *pFastBacI-PMS2*. *pFastBacI-His$_6$-hPMS2 [D70N]:* An *Afl*II/*Xba* I fragment of vector *pFastBacI-hPMS2[D70N]* was cloned between the *Afl*II/*Xba*I sites of *pFastBacI-His$_6$-hPMS2.*

All constructs were sequenced over the entire length of the sub-cloned PCR fragments and checked for the presence of the desired base substitutions.

Oligonucleotides

mr_2: (5'CGGAATTCTATCTGTATGCACACTTTCCAT3')

mr_4 : (5'CGGGATCCAAGCTCGTTCGTGGCAGGGGTT3')

mr_18 : (5'ATGCTATCAAAGCGATGATTGAGAAC3')

mr_19 : (5'CAGTTCTCAATCATCGCTTTGATAGC3')

mr_20 : (5'GAGATGATTGAGGCCTGTTTAGATGC3')

mr_21: (5'GCATCTAAACAGGCCTCAATCATCTC3')

mr_22: (5'GCGGTAAAGGCGTTAGTAGAAAACAG3')

mr_23: (5'CTGTTTTCTACTAACGCCTTTACCGC3')

mr_24: (5'GGAGTTAGTAGAAGCCAGTCTGGATGC3')

mr_25: (5'GCATCCAGACTGGCTTCTACTAACTCC3')

mr_26: (5'GCTGCACGCTGACTGTGG3')

mr_34: (5'GATTATTCATACCGTCCCACC3')

mr_38: (5'CAGATCCAAAACAATGGCACC3')

mr_39: (5'GGTGCCATTGTTTTGGATCTG3')

mr_40: (5'GAAGTTTCAAACAATGGATGTGG3')

mr_41: (5'CCACATCCATTGTTTGAAACTTC3')

mr_42: (5'CACACACGGAGTCACTAGGG3')

mr_55: (5'GCGGATCCACCATGTCGTACTACCATCACCATCACCATCACGATTACGATATCCCAACGACCGAAAACCTNGTA TTTTCAGGGCGAATTCGAGCGAGCTGAG3')

48_Homo: (5'CCCAGTTGCACCCGTTTCTGGAGCCACGTTCGGTCTTAACCCGTCCAG3')

48_Δ1: (5'CCCAGTTGCACCCGTTTCTGGAGC**A**CACGTTCGGTCTTAACCCGTCCAG 3')

48_Bottom (5'CTGGACGGGTTAAGACCGAACGTGGCTCCAGAAACGGGTGCAACTGGG 3')

81BG -GTGT-: (5'GACACAGGAAACAGCTATGACCATGATTACGCCAAGCTTGGCTGCAGGTTGTGCGACGGATCCCACTAGCCC AACTCATCC3')

Overexpression of the hMutLα Variants- Typically, $1.8x10^8$ *Spodoptera frugiperda* (Sf9) cells were infected with a combination of two viruses encoding the *hMLH1* and *hPMS2* variants at a MOI of 10. 72 h after infection, cells were harvested by centrifugation (1500 rpm, 10 minutes). After washing the cells with 10 pellet volumes of cold PBS containing 0.5 mM PMSF, the cells were resuspended in 3 pellet volumes of cold buffer A (25 mM HEPES pH 8.0, 2 mM 2-mercaptoethanol, 0.5 mM spermidine, 0.15 mM spermine, 0.5 mM PMSF and 2X Complete$^{EDTA\ free}$ (Roche). After incubation (20 minutes, 4 °C), the cells were lysed in a Dounce homogenizer (15 strokes). 2 pellet volumes of a cold 50 % glycerol solution and NaCl (to a final concentration of 300 mM) were added. After incubation (30 minutes, 4 °C, mixing), extracts were centrifuged (36000 rpm, 30 minutes, 4 °C in a Sorvall TH-641 rotor), and stored in aliquots at 80 °C.

Purification of the hMutLα Variants- Extracts from infected Sf9 cells were diluted with an equal volume of buffer H (25 mM HEPES pH=7.6, 2 mM β-ME) and centrifuged (36000 rpm, 30 minutes, 4 °C in a Sorvall TH-641 rotor). Filtered extracts (0.45 μm) were applied to a 5 ml Heparin HiTrap column (AP Biotech) equilibrated with a buffer H containing 150 mM NaCl and proteins were eluted with a raising 2 %/ml salt gradient. Pooled fractions containing the hMutLα variants were adjusted to 5 mM imidazol. An appropriate volume of Ni-NTA agarose (STRATAGENE) (approximately 7 ml of a 50 % slurry per 100 mg extracts from Sf9 cells expressing wild-type hMutLα) was added. After incubation on an end-over-end shaker for 60 minutes at 4 °C, extracts were transferred into dispensable plastic columns (Bio-Rad). The columns were washed five times with 5 column volumes of cold buffer WB (300 mM NaCl, 10 % glycerol, 20 mM HEPES pH 8, 2 mM 2-mercaptoethanol, 200 mM PMSF) containing 10 mM imidazol. Bound proteins were eluted with 5 column volumes of buffer WB containing 100 mM imidazol and with 5 column volumes of buffer WB containing 200 mM imidazol. Fractions containing the highly purified MutLα variants were pooled, adjusted to 150 mM NaCl and concentrated on a 1 ml Resource-Q (AP Biotech) column.

Prior to the injection into the Resource-Q column, proteins were passed through a 6 ml Resource-S column to remove possible contaminants. After injection, the Resource-S column was removed and proteins were eluted with a raising 4 %/ml salt gradient. The fractions containing the pure proteins were pooled, dialysed extensively against storage buffer (20 mM HEPES pH=7.6, 0.1 mM EDTA, 110 mM NaCl, 10 % sucrose, 2 mM 2-mercaptoethanol and 0.5 mM PMSF), and stored in aliquots at -80 ÚC.

Other Proteins- The purified hMutSα was described previously (43,44).

UV Cross-l inking- An aliquot of purified protein (1 µg) was incubated for 30 minutes at 4°C in 10 µl crosslinking buffer (50 mM Tris-HCl (pH=7.5), 6 mM $MgCl_2$, 10 % glycerol, 10 % sucrose, 5 mM DTT, 0.2 mg/ml BSA and 0.5 µM [α-^{32}P]-ATP). The samples were placed on ice, irradiated for 5 minutes in an UV-Stratalinker (STRATAGENE) and separated by 7.5 % SDS-PAGE. Gels were extensively washed in fixing solution (30 % methanol, 10 % acetic acid) and analyzed by autoradiography.

Partial Proteolysis Experiments- In a final volume of 20 µl, 3 µg (16.5 pmol) of the recombinant proteins were with 25 mM TrisCl (pH=8), 120 mM NaCl, 1 mM DTT in the presence or absence of 1 mM $MgCl_2$. The ATP concentration was between 0 and 10 mM, as shown in Fig. 6. After a 15 minute pre-incubation at 20 ÚC, 10 ng of trypsin (Promega) were added and the reaction was allowed to proceed for 8 minutes at 20 ÚC. The reactions were stopped by the addition of 4 µl 6x SDS loading dye and boiling for 5 minutes at 95 ÚC. The samples were loaded onto 10 % SDS polyacrylamide gels and the protein bands were visualised with Coomassie Blue.

ATPase Assays- The ATPase assays were carried out at 37 °C in a 15 µl reaction mix containing 20 mM TrisCl (pH=8), 120 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 400 µM cold ATP, 222 nM [γ-^{32}P]-ATP and 60 pmol purified protein (4 µM). To test the DNA-dependent stimulation of the hMutLα heterodimer, either an 81-mer single-stranded oligonucleotide (*81BGTGT*; final

concentration 12 μM) or a 3193 nucleotide long single-stranded and covalently closed phagemid DNA (*pGEM-T*; final concentrations 2, 20, or 200 nM) were added to the reactions (data not shown). At selected time points, 2 μl aliquots were removed and mixed with 5 μl formamide loading dye. 2 μl aliquots were loaded onto 20 % denaturing polyacrylamide gels, which were then exposed to Biomax MR films (KODAK). The bands were quantified using the ImageQuant Software v1.2 (MOLECULAR DYNAMICS, INC.). For the calculation of the Michaelis-Menten constants, the ATPase activity was measured in the presence of varying concentrations of cold ATP (200-1000 μM).

In vitro MMR Assays – The MMR assays were carried out as described previously (45)159}. Briefly, cytoplasmic protein extracts were prepared from TK6 and HCT116 cell lines, using $5x10^8$ cells harvested in the exponential growth phase. After resuspension in ice-cold hypotonic buffer (20 mM HEPES pH=7.9, 5 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM PMSF, 1 mM DTT) at a density of $1x10^8$ cells/ml, the cells were allowed to swell for 10 minutes in a glass Dounce homogenizer on ice, and then lysed mechanically by applying four or more strokes with a tight pestle. When more than 80 % of cells were lysed, the nuclei were pelleted, the supernatant was centrifuged (12000 x g for 10 minutes at 4 ÚC) and stored in small aliquots at -80 ÚC.

Wild type and mutant M13mp2 phage used to generate the mismatch-containing heteroduplexes were kindly provided by Tom Kunkel. The heteroduplex DNA contained the indicated base/base mispair within the coding sequence of the lacZ α-complementation gene, and a nick either 5 or 3 from the mispair. 1 fmol of substrate was incubated with 50 μg of cytoplasmic extract, supplemented where necessary with 200 ng of the purified recombinant hMutLα variants. The repair reaction (25 μl) contained 30 mM HEPES pH=7.8, 7 mM $MgCl_2$, 4 mM ATP, 200 μM each CTP, GTP and UTP, 100 μM each dATP, dCTP, dGTP and dTTP, 40 mM creatine phosphate, 100 fmol creatine phosphokinase and 15 mM sodium phosphate pH=7.5. After 20 minutes of

incubation at 37 ÚC, the heteroduplex DNA was purified and electroporated into *E.coli* NR9162 (mutS), plated on minimal medium in a soft agar layer containing 0.5 ml of a log culture of E.coli CSH50, 0.5 mg IPTG and 2 mg X-Gal. After incubation for 16 h at 37 ÚC, repair efficiency could be determined by analyzing the color of the plaques.

Bandshift Assays- The bandshift experiments were performed with either a 48mer heteroduplex oligonucleotide containing a 1 base insertion loop (Δ1) or a homoduplex G/C. The substrates (80 fmol, 4 nM), hMutSα (75 nM) and hMutLα variants (150 nM) were incubated for 20 minutes on ice in 20 µl binding buffer (20 mM HEPES pH=7.6, 1 mM DTT, 50 µg/ml BSA, 120 mM NaCl, 1.75 ng/µl poly(dI-dC)•poly(dI-dC) (AP Biotech), 12.5 % Glycerol). If indicated, 2 mM $MgCl_2$ and/or 0.5 mM ATP were added prior to the addition of the proteins. 2 mM EDTA was added to reactions, in which $MgCl_2$ was omitted. If indicated, monoclonal antibodies against hMSH6 (Serotec, 66H6, 0.2 µg/µl) or hMLH1 (Pharmingen, cat. 13291A, 0.05 µg/µl) were added 10 minutes after the start of the incubation. 5 µl of each sample was carefully loaded onto a 4 % non-denaturing polyacrylamide gel (acrylamide-bisacrylamide 37.5:1). Gels were run in TAE buffer (pH=7.5) at 4 °C, and ^{32}P-labeled protein-DNA complexes were visualized using a STORM phosphorimager (Molecular Dynamics Inc.). The homoduplex and Δ1 substrates were constructed by annealing the oligonucleotide *48_Homo* or *48_Δ1* with the complementary strand (*48_Bottom*) that was labeled at its 5' end with ^{32}P using the polynucleotide kinase.

RESULTS

Rationale for mutational analysis of the ATP binding domains of hMutLα↑ Comparison of the primary amino acid sequences and the available crystal structures of the nucleotide binding domains of MutL, NgyrB and HSP90 revealed that the ATP binding sites, including all known catalytic residues, are highly conserved (16,19,46). According to the GHKL consensus motifs

(13,14), three invariant amino acids were selected for mutagenesis (Fig. 1a, red stars). Their choice was based on the recently determined structure of the amino terminal fragment of MutL in complex with the non-hydrolyzable ATP analogue ADPNP, which revealed the distinct contributions of these residues towards the ATPase function (16) (Fig. 2). In MutL, the base of the ATP nucleotide is bound in a deep hydrophobic pocket. The first residue, a polar Asp-58 lies at the bottom of this pocket and forms a direct hydrogen bond with the exocyclic amino group of the adenine base. In HSP90, substitution of the equivalent aspartate by alanine or asparagine completely abolished nucleotide binding and the *in vivo* function of the protein (21,47). The second residue, Asn-33 plays a crucial role in the binding of the Mg^{2+} ion, because its side chain contributes both directly and *via* a water molecule to the coordination of the metal ion. In GHLK ATPases the nucleotide adopts a kinked conformation and oxygen atoms from all three phosphates contribute to the coordination of the metal. Accordingly, MutL and HSP90 are unable to bind ATP in the absence of Mg^{2+} (16,19) and substitution of the equivalent asparagine residue in HSP90 completely abolished its ATPase activity (21). The third residue, Glu-29 is most likely involved in the activation of the catalytic water molecule. In agreement with its proposed role as a general base, substitution of this residue with alanine abolished the ATPase activity of MutL (16,17), HSP90 (47,48), and type II topoisomerases (18,49) with only little or no effect on the binding of the nucleotide.

In order to study the role of ATP binding and/or hydrolysis in the function of hMutLα, we used site-directed mutagenesis to generate variants of hMLH1 or hPMS2 that carried single amino acid substitutions in one of the three above sites (Fig. 2). The hMutLα variants studied here are the following: the ATP binding deficient double mutant hMutLα [hMLH1(D63N)/hPMS2(D70N)], designated $L\alpha^{DN/DN}$, the Mg^{2+} binding deficient double mutant hMutLα [hMLH1(N38A)/hPMS2(N45A)], designated $L\alpha^{NA/NA}$, and the catalytic site double mutant hMutLα [hMLH1(E34A)/hPMS2(E41A)], designated $L\alpha^{EA/EA}$. $L\alpha^{DN/WT}$ and $L\alpha^{WT/DN}$ indicate mixed

heterodimers where either the hMLH1 or the hPMS2 subunit contains the relevant substitution, respectively.

Expression of hMutLα can be severely affected by single amino acid substitutions in the ATP binding domains of its subunits - Detailed analysis of the crystal structure of the N-terminal MutL fragment or of modeled structures of the hMLH1 ATP binding domain suggested that none of the planned substitutions should affect the overall protein fold. We were therefore surprised to find that some of the amino acid changes, such as the substitution of Asp-63 in the ATP binding pocket of hMLH1(D63N), resulted in a dramatically reduced expression of the hMutLα variants containing this subunit (Fig. 3, lanes 2,4). This effect was caused presumably through destabilization of the mutated hMLH1 and the consequent degradation of hPMS2, which is labile in the absence of the hMLH1 subunit (37). Interestingly, the equivalent substitution (D70N) in the hPMS2 subunit had no apparent effect on protein expression (lane 3). Sequencing of the expression constructs and detection of the full-length hMLH1 protein in these extracts by Western blotting ruled out the possibility that the reduced expression was due to additional mutations, which might have caused the premature truncation of the proteins (data not shown). In order to rule out an artifact inherent to the baculovirus system, we also analyzed this substitution in a homologous expression system, based on human 293T cells, which do not express hMLH1 (50). Co-transfection of these cells with vectors encoding either wild type or mutant hMLH1 and hPMS2 proteins also lead to a drastic reduction of the expression levels of hMutLα, when the hMLH1 subunit carried the D63N substitution (data not shown).

Mutation of the asparagine involved in coordination of the Mg^{2+} ion had a deleterious effect on the expression of hMutLα only if both subunits carried the alanine substitution in their ATP binding pockets (Fig. 3, lane 7). Because identical titers of the baculovirus encoding hMLH1(N38A) were used for the parallel production of $L\alpha^{NA/WT}$ and $L\alpha^{NA/NA}$, the difference in expression levels cannot be attributed to differences in the multiplicity of infection or in the quality of the insect cells used in the experiment. Our results suggest, therefore, that ATP binding by hMLH1 and, to a lower

extent also by hPMS2, is critical for the stable expression of the hMutLα heterodimer.

In contrast to the mutants described above, substitution of the catalytic residues in hMLH1 (E34A) or in hPMS2 (E41A) affected neither the expression nor the stability of the proteins (Fig. 3, lane 8-9). Given that these latter mutants still bind ATP [ref. (51), see also Fig. 6] our results suggest that ATP binding, but not hydrolysis, is required for the stabilization of hMutLα.

Purification of the hMutLα variants- In order to facilitate the purification of the hMutLα variants, all hPMS2 expression vectors were modified to include an amino-terminal $(His)_6$ tag. These vectors were co-expressed with the untagged hMLH1 in Sf9 cells and the resulting heterodimers were purified by a three-step procedure including chromatography on Ni-NTA (Fig. 4, see Experimental Procedures). The fact that the untagged hMLH1 variants co-purified with the $(His)_6$-hPMS2 variants in an equimolar ratio implies that the ATP binding domains are unlikely to be involved in the regulation of the stable dimerization of the carboxy-terminal domains of hMLH1 and hPMS2 (36,38). However, the poor expression of the hMLH1(D63N) variant precluded the purification of the $L\alpha^{DN/WT}$ and $L\alpha^{DN/DN}$ heterodimers. In addition, the $L\alpha^{NA/NA}$ variant was expressed in low amounts and could not be purified to the same extent as the others. This protein preparation contained a contaminant, which migrated slightly slower than hMLH1 in SDS-PAGE (Fig. 4). Although the contaminating protein did not appear to interfere with our assays, the results of experiments in which it was used were interpreted with caution.

ATP cross-linking reveals an inherent asymmetry of the two ATP binding sites of hMutLα - We used UV cross-linking to study the ATP binding properties of the hMutLα variants. Surprisingly, while the hMLH1 subunit of the wild type hMutLα complex was efficiently cross-linked to [α^{32}P]ATP, only little radioactivity was found associated with the hPMS2 subunit (Fig. 5). In agreement with the extensive interaction between the three phosphate groups of the nucleotide and the Mg^{2+} ion observed in all structures of the GHLK ATPases, UV crosslinking of ATP to the hMLH1 subunit

showed an absolute requirement for Mg^{2+}. Correspondingly, substitution of the Mg^{2+} co-ordinating asparagine with alanine greatly reduced ATP cross-linking efficiency to the hMLH1 (N38A) mutant (Fig. 5). These results indicate that hMLH1 and hPMS2 bind the nucleotide with substantially different affinities, and could be explained in two ways. Either by the binding of the nucleotide in the hMLH1 subunit and not in hPMS2, or by the substantially longer residence times of the bound ATP molecule within the nucleotide binding pocket of hMLH1, which results in more efficient cross-linking. In an attempt to understand which of these two possibilities applies, we carried out partial proteolysis experiments and ATP hydrolysis assays.

ATP binding brings about a conformational change in hMLH1 that is detectable by partial proteolysis We incubated the wild type hMutLα heterodimer with or without ATP in the presence of trypsin. In the absence of the nucleotide, the two polypeptides were degraded to numerous peptide fragments, ranging from ~60 to ~33 kDa and this pattern was retained also in the presence of low (0.1 and 1 μM) ATP concentrations (Fig. 6a). However, in the presence of 10 μM ATP a novel band of about 39 kDa appeared (Fig. 6, arrowed) and persisted up to an ATP concentration of 10 mM. Interestingly, no other major differences were detected in the peptide pattern. This suggests that one of the hMutLα subunits bound ATP at a concentration of ~10 μM and above, and that this binding event resulted in a conformational change, which altered its proteolytic pattern. The results of the ATP cross-linking experiments suggested that the polypeptide in question should be hMLH1. This was confirmed through the partial proteolysis of the $L\alpha^{NA/wt}$ and $L\alpha^{wt/NA}$ variants. The latter heterodimer produced a tryptic peptide pattern that was identical to the wild type hMutLα. In contrast, the $L\alpha^{NA/wt}$ variant, in which the ATP binding site of MLH1 was mutated, failed to give rise to the 39 kDa proteolytic band (Fig. 6b). We were able to confirm the identity of the band also in LC/MS/MS experiments (data not shown), where it was unambiguously assigned to the N-terminus of hMLH1, as one of its tryptic peptides corresponded to amino acid residues 10-18 (RLDETVVN)

of this protein.

As can be seen in Fig. 6b, the 39 kDa proteolytic fragment of hMLH1 only appears in the presence of both ATP and Mg^{2+}. This finding further confirms that the binding of the nucleotide is absolutely dependent on the presence of the divalent metal cation, as was shown for MutL (16) and HSP90 (19). The partial proteolysis experiments further showed that the EA mutation in the catalytic site of the hMLH1 subunits does not affect nucleotide binding, as the ATP-dependent 39 kDa tryptic band was present in all three variants, $L\alpha^{WT/WT}$, $L\alpha^{EA/WT}$ and $L\alpha^{EA/EA}$ (Fig. 6c).

The ATPase activity of hMutLα is very low and is not stimulated by single stranded DNA The ATPase activity of the highly-purified wild type hMutLα is very low. Our semi-quantitative estimates put the ATPase activity of wild type hMutLα in a similar range as that of MutL, i.e. ~0.5 min^{-1} (16,24) and our data not shown. As anticipated, the ATPase activity of the $L\alpha^{EA/EA}$ heterodimer was very close to background. Interestingly, mutations in the ATP catalytic site of hMLH1 and hPMS2 had different effects. Thus, while the hMLH1 mutant $L\alpha^{EA/wt}$ displayed an ATPase activity that was intermediate between the wild type and the double mutant factors, the activity of the $L\alpha^{wt/EA}$ variant was severely attenuated, being close to that of $L\alpha^{EA/EA}$ (Fig. 7).

In contrast to its prokaryotic homologue (17), the ATPase activity of the wild type hMutLα complex was not stimulated by the addition of single-stranded DNA (81-mer oligonucleotide or 3.2 kb phagemid; data not shown).

Substitutions in the ATP binding domains of hMLH1 and hPMS2 affect the biological activity of hMutLα in in vitro MMR assay to similar extents We tested the biological activity of the hMutLα variants in an *in vitro* MMR assay, in which the recombinant proteins were used to complement the MMR-deficient extract of HCT116 cells that lack hMutLα (37). As shown in Fig. 8, no repair of heteroduplex plasmid substrates containing a nick located either 5 or 3 from a single GT mismatch was observed after 20 minutes of incubation with the HCT116 extracts. In contrast, more

than 80% of the substrate were repaired if wild type hMutLα (200 ng) was added to these extracts. Titration experiments revealed that the MMR complementation efficiency of the His-tagged hMutLα was quantitatively indistinguishable from that of an untagged hMutLα complex (data not shown and (37)). Moreover, since removal of the His-tag by the TEV protease did not change the complementation efficiency of the heterodimer (data not shown), we concluded that the presence of the His-tag on the hPMS2 subunit did not have any effect on the biological activity of hMutLα.

Substitution of the catalytic glutamate residue affected the *in vitro* MMR activity of hMutLα to different extents. Thus, while the catalytic double mutant was inactive in the complementation assay, the variants carrying the substitution in only one subunit displayed intermediate levels of MMR activity. Importantly, repair from both the 5 or 3' direction was affected to a similar extent, regardless of which subunit carried the substitution. These results clearly demonstrate that although the ATPase activities of both subunits are required for efficient MMR, the process can still function, albeit less efficiently, when only one subunit can hydrolyze the nucleotide. This was not the case when the ATP binding ability of the hMutLα subunits was affected; in this case, already a single subunit mutation resulted in the loss of complementation activity, which implies that ATP binding by both subunits is required for MMR.

ATP stimulates the formation of a ternary complex between hMutLα, hMutSα and DNA - The interaction between purified hMutLα and hMutSα on mismatched DNA was investigated in bandshift assays using a 48-mer homoduplex (G/C), or a similar 48/49-mer heteroduplex substrate containing a single nucleotide insertion in one strand (Fig. 9, *left panel*). In the presence of hMutSα, a specific protein/DNA complex (*) was formed, which dissociated upon the addition of 0.5 mM ATP or ATP"Mg^{2+} (see also (43)). However, while ATP brought about the dissociation of hMutSα from the oligonucleotide substrate, it promoted the formation of a ternary complex (**) between the oligonucleotide, hMutSα and hMutLα. This species appears to contain both heterodimers, as addition of anti-hMSH6, anti-hMLH1 or anti-hPMS2 antibodies resulted in a further retardation of the

complex (***). Interestingly, formation of the ternary complex was dependent on the presence of ATP, but not magnesium. As hMutLα does not undergo an ATP-driven conformational change in the absence of the metal ion (Fig. 6b), we concluded from these experiments that interaction of the hMutSα and hMutLα heterodimers requires ATP binding, but not hydrolysis, in the hMutSα factor. These results support and extend the findings reported for the *S. cerevisiae* MutSα and MutLα homologues (52-54).

Mutations in the ATP binding domains of hMutLα do not affect the formation of the ternary complex with hMutSα on DNA The ATP-dependent interaction between hMutLα and hMutSα observed in the bandshift experiments might reflect the recruitment of hMutLα to the hMutSα-bound mispair at a very early step during the repair reaction. We were therefore interested to see whether the *in vitro* complementation defect of the hMutLα variants was caused by their inability to interact with the hMutSα complex. As shown in Fig. 9 (*right panel*), all of the hMutLα variants that we were able to purify in sufficient amounts were able to form the ternary complexes (**), which suggests that the nucleotide dependence of the ternary complex formation is not dictated by the hMutLα heterodimer. (NB: The supershifted band in lane 7 of the right panel of Fig. 9 due to $L\alpha^{NA/NA}$ is weaker than that observed in the other lanes. This is not due to the reduced ability of the protein to form a ternary complex, but rather to the lower amounts of the heterodimer used in this experiment. See also legend to Fig. 9.) Taken together, our results suggest that the deleterious effect of the substitutions in the ATP binding sites of hMutLα is linked to a step downstream from the recruitment of the heterodimer to the DNA-bound hMutSα complex.

DISCUSSION

Despite the identification of several MutL homologues in the human genome, the hMLH1/hPMS2 heterodimer hMutLα plays the predominant role in postreplicative MMR. This is witnessed primarily by the fact that human tumor cell lines carrying mutations in either gene have mutator phenotypes of similar magnitude (32,34). As this shows that both subunits of hMutLα are equally important for MMR, the finding that the *hPMS2* gene was only extremely rarely mutated in HNPCC families was unexpected and implied either that the two genetic loci are differentially susceptible to mutagenesis, or that mutations in *hPMS2* are phenotypically silent. The results of our present investigation tend to support the former hypothesis, as they revealed major differences in the effects that the seemingly equivalent mutations had on the two polypeptides. Thus, the conservative substitution of an aspartate by an asparagine within the NBDs of hMLH1 and hPMS2 was anticipated to have no effect on the folding of the polypeptides. This expectation was based on the finding that the HSP90(D93N) mutant could be shown to retain its structural fold (47) and was substantiated in the case of the hMutLα variant carrying the D70N mutation in the ATP binding domain of hPMS2, which was expressed with an efficiency similar to wild type. Unexpectedly, the D63N mutation that abolished ATP binding in the hMLH1 subunit deleteriously affected the expression efficiency of the heterodimer (Fig. 3), similarly to observations made with hMutLα variants carrying missense mutations identified in HNPCC families that mapped close to the ATP binding site of hMLH1 (Fig. 1b,(50,52,55)). This effect may be linked with an increased susceptibility of the mutated protein to degradation, as suggested by experiments where ATP binding was shown to protect the amino terminus of the *S. cerevisiae* MLH1 against proteolysis (51). These results demonstrated that the ability to stably bind ATP is essential for the stability of hMLH1 but not hPMS2, and implied that the two polypeptides may have different affinities for the nucleotide, despite the conservation of their NBDs. This latter prediction could be substantiated by the greater

efficiency of [α-^{32}P]-ATP cross-linking to hMLH1 (Fig. 5). We postulate that the ATP binding site of hMLH1 needs to be occupied at physiological ATP concentrations, and thus that it is adversely affected by the absence of the nucleotide, while the ATP binding deficient variant of hPMS2 would not be destabilized, as this protein does not appear to require bound ATP for correct folding or stabilization against proteolysis. Given the importance of ATP binding to hMLH1 stability, it was surprising to see that the Lα$^{NA/WT}$ variant was expressed at levels similar to the wild type protein. This phenomenon could be explained if we assume that the affinity of this variant for ATP was strongly reduced, but not eliminated. In this scenario, the low level of ATP binding observed in the UV cross-linking experiments (Fig. 5) might be sufficient to stabilize the protein, but too low to support its biological activity (see also below).

Interestingly, while the ATP cross-linking (Fig. 5) and partial proteolysis (Fig. 6) experiments implied that the hMLH1 subunit of hMutLα bound the nucleotide with higher affinity, attenuation of ATP binding in the hPMS2 subunit affected the ATPase activity of the heterodimer to a greater extent than did the same mutation in the hMLH1 subunit. These findings suggest that hPMS2 is catalytically more active than hMLH1 and that the ATP residence times of the nucleotide in the ATP binding pocket of hPMS2 might be too short for efficient cross-linking or for effective protection from trypsin. These findings are supported by those of Yang and colleagues (56), who demonstrated that the isolated N-terminal domain of hPMS2 has ATPase activity.

In order to test how the observed differences in ATPase activities of the two hMutLα subunits affected the biological activity of the hMutLα variants under study, we tested the recombinant factors in *in vitro* MMR assays. As anticipated, the Lα$^{EA/EA}$ and Lα$^{NA/NA}$ double mutants that were unable to hydrolyze or bind ATP, respectively, were unable to complement the hMutLαdeficient extracts of HCT116 cells (Fig. 8). The same was true for the single subunit mutants that were deficient in nucleotide binding. These results show that ATP binding in both subunits is

essential for MMR. Surprisingly, the $L\alpha^{WT/EA}$ and $L\alpha^{EA/WT}$ hMutLα variants, in which the hydrolytic capacity was attenuated in one or the other subunit, displayed significant, but similar reductions in MMR efficiency. This result was unexpected, given that the ATPase activities of the two heterodimers were affected by the respective mutations to different extents, and implied that hMutLα must be able to hydrolyse ATP in order to be active in MMR, but that the activity can be extremely low (Fig. 8). Interestingly, we made a similar observation when we mutated the ATP binding sites of the hMSH2 and hMSH6 subunits of hMutSα (43). While the latter subunit contributed more towards the ATPase activity of the heterodimer, the effect of mutations in hMSH2 and hMSH6 on mismatch repair efficiency *in vitro* was similar.

While this study was in progress, the mutator phenotypes of knock-in *S. cerevisiae* strains carrying mutations within the ATPase domains of MLH1 or PMS1 (the functional homologue of hPMS2) were described (51). The substitutions altered amino acids corresponding to the catalytic glutamates or to invariant glycine residues located in the respective phosphate binding loops of the proteins (Motif III in Figs. 1 and 2), and were predicted – similarly to our study – to abolish nucleotide hydrolysis or nucleotide binding, respectively. In agreement with our results, the strain in which both genes carried a substitution of the catalytic glutamate residue displayed a strong mutator phenotype, while mutation of only a single MutLα subunit produced a weaker mutator effect. However, in the yeast system, the *mlh1* single mutant was more affected than the corresponding *pms1* variant, and this functional asymmetry became even more pronounced for mutants that carried the glycine to alanine substitutions: the *mlh1-G98A* strain showed a full mutator phenotype comparable to a MMR-deficient strain, while the *pms1-G128A* strain was only a very weak mutator. This latter finding cannot be easily explained. Our results showed that ATP binding is more important than ATP hydrolysis and similar data were reported for "heterodimeric" topoisomerase II variants (57-59) mentioned above. Thus, assuming that the G128A mutation in the P-loop motif of the *S. cerevisiae* protein abolished ATP binding, the mutator phenotype of the *pms1-G128A* strain

would have been expected to be stronger than that of the PMS1 mutant deficient in ATP hydrolysis. The discrepancy between our results and those generated by Liskay and colleagues suggests that mutation of the conserved glycine within the P-loop of the yeast PMS1 protein gave rise to a complex phenotype, which cannot be explained simply by the loss of nucleotide binding.

The functional asymmetry observed with the yeast MutLα was suggested to reflect an unequal contribution of the two subunits towards the repair events with a 5→3 or a 3→5 directionality (51). Such an interpretation would require the two MutLα subunits to interact with different downstream factors and the two ATP binding domains to act independently of each other. We addressed this question by comparing the repair efficiencies of the single mutants on substrates that contained the nick either on the 5' or the 3' side of the mismatch. The finding that these substitutions affected MMR from both directions to a similar extent implies that the above hypothesis does not apply to the human system and that the two ATP binding domains of hMutLα act in an interdependent way. Evidence for such a coordinated function is provided by genetic analysis (60) and by elegant two hybrid experiments carried out with the N-terminal fragments of yeast MutLα (51), which clearly showed that ATP binding at both subunits is required in order for the nucleotide binding domains to dimerize. Assuming that the carboxy-termini of the full-length proteins remain stably associated, the function of the amino-termini could be to form an ATP-driven molecular clamp, which then interacts with the downstream MMR factors.

Taken together, the data presented in this study allowed us to propose a model for hMutLα function, in which ATP binding operates a molecular clamp formed by the NBDs of its two subunits. The ATP binding site of the hMLH1 subunit might be mostly occupied, while that of the hPMS2 subunit might oscillate between the bound and the unbound form and thus ensure that the clamp does not close stably. Interaction with DNA and/or other MMR proteins (e.g. mismatch-bound hMutSα) might alter the nucleotide binding properties of the ATP binding pocket of the hPMS2 subunit, which could result in closing of the clamp and in immobilization of hMutLα at its site of action. The

heterodimer might then be recycled by the hydrolysis of the nucleotides, which would lead to opening and release of the clamp. This mode of action would explain the MMR phenotype of all our mutants. While variants unable to bind ATP at one or both of their subunits would fail to close the clamp, the catalytic double mutants might be MMR deficient, because their clamp would remain closed. The partial defect in MMR of the single catalytic mutants could also be explained if we assume that ATP hydrolysis catalyzed by only one subunit might be sufficient to induce the dissociation of the amino-termini and thus to recycle the repair factor, but more slowly.

The biological role of the MutL homologues has been frequently described as that of "molecular matchmakers". Studies carried out with the prokaryotic MutL suggest that it functions as a bridging molecule that transmits signals from the mismatch-bound MutS to the downstream factors. MutL could be shown to interact with the DNA helicase II and to load it at the site of the nick, where the process of exonucleolytic degradation of DNA is initiated. This nick was introduced into the DNA by MutH, the endonucleolytic activity of which could be correspondingly shown to be activated by MutL in a reaction that requires ATP binding, but not hydrolysis (4,16). This activation is even more efficient in the presence of MutS and a mismatched substrate, but in this case it requires ATP hydrolysis. That the ATPase activity of MutL is involved was shown by the inability of the MutL mutant (E29A), which binds ATP but fails to hydrolyze it, to bring about the full, MutS-dependent activation of MutH (4,5,16,61). Although the mechanism of strand discrimination in eukaryotes differs from that of *E. coli*, the role of the eukaryotic MutL homologues lies most likely in controlling protein/protein interactions, as in the prokaryotic system. In eukaryotic cells, biochemical studies suggested that MutSα and MutLα interact on DNA in an ATP-dependent manner (53,62,63). The human factors are capable of similar interactions (Fig. 9 and (64)). Interestingly, our study shows that because the ternary complexes were formed between hMutSα and all the hMutLα variants, their formation could not be driven by ATP binding within the hMutLα component. This prediction was further substantiated by the formation of the MutSα"MutLα"DNA

complexes in the absence of magnesium, i.e. under experimental conditions where hMutLα does not bind ATP. It would therefore appear that the association of hMutSα, hMutLα and mismatched DNA is driven by ATP binding within hMutSα, as this heterodimer was shown previously to undergo an ATP-driven conformational change that was independent of nucleotide hydrolysis (44). Although the supershift experiments leave many questions open, they show that the hMutLα variants studied here retained their overall structures, such that they were still able to interact with DNA-bound hMutSα. We therefore conclude that the observed lack of activity of these proteins in our *in vitro* MMR assay must be due to their failure to interact with components of the MMR machinery that are downstream from mismatch recognition, such as PCNA and/or EXO1 (54,60).

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FIGURE LEGENDS

FIG. 1. **a, Conserved sequence motifs that constitute the ATP binding pockets of GHL ATPases.** *Left panel:* Structure-based alignment of the amino-terminal sequences of MutL (*E.coli*), hMLH1, hPMS2, Gyrase B (*E.coli*) and human HSP90 reveals the presence of four highly-conserved motifs

(I-IV, red). Red stars indicate amino acid residues that were substituted in this study. The secondary structures of the nucleotide-bound proteins are schematically drawn above the sequence alignment. *Right panel:* Ribbon diagram of the MutL (1-349) crystal structure in complex with ADPNP. The polypeptide folds into two globular domains (amino acids 20-220 and 224-331). Motifs I-IV (red) mark the site of ATP binding within the first domain (see also Fig. 2). Note that only one subunit of the dimeric protein complex is shown. (The figure was prepared using file 1b63 from the Brookhaven Protein Data Bank (PDB) (17)). **b,** Clustering of HNPCC germline mutations in the *hMLH1* gene. All *hMLH1* single amino acid substitutions reported in the HNPCC database were aligned along the primary sequence of *hMLH1*. Amino acid substitutions shown in red gave rise to low expression in other functional assays (50,52,55). (?) indicates that the pathogenicity of the substitution is uncertain, due to incomplete segregation data or because the variant was proficient in a functional assay. Green boxes indicate regions conserved in all MutL homologues. Red boxes indicate the four conserved motifs that constitute the ATP binding pocket of hMLH1. Blue boxes indicate regions that are conserved among eukaryotic *MLH1* homologues. These regions have been implicated in the interaction with the respective *PMS* homologues (36,38).

FIG. 2. **ATP binding pocket of MutL.** Amino acids from the four conserved motifs (I-IV, red) are shown in ribbon representation. *Left panel:* Structure of the nucleotide-free protein. Helix A (Motif I) harbors the catalytic base (Glu-29) and the asparagine residue (Asn-33) that is involved in the coordination of the Mg^{2+} ion. Motif II and IV are part of an extended β-sheet. Asp-58 sticks out from this β-sheet to form a H-bond with the bound nucleotide. Note that helix D (Motif III) occupies the ATP binding pocket and has to unfold in order for the nucleotide to gain access to its binding site. *Center panel:* Structure of the protein in complex with ADPNP. In the presence of the nucleotide, the amino-terminal part of helix D unfolds to form a flexible loop involved in the binding of the phosphate groups (P-Loop). ADPNP and Mg^{2+} are shown in gray and green,

respectively, and water molecules are shown as blue spheres. *Right panel:* Amino acid substitutions introduced into hMLH1 and hPMS2, respectively. (The figure was prepared using the files 1BKN *(left)* and 1b63 *(center)* from the Brookhaven PDB).

FIG. 3. **Expression of hMutLα variants in baculovirus-infected Sf9 cells.** Sf9 cells were co-infected with baculovirus vectors encoding either wild type or mutant forms of the untagged hMLH1 and hPMS2 proteins as indicated above the panel (see text for abbreviations). Total cell extracts (20 μg) were the separated on a 7.5 % SDS polyacrylamide gel and stained with Coomassie Blue. Note that a very abundant band migrated with the same size as hMLH1 (compare extracts of infected vs. non-infected cells shown in lane 10).

FIG. 4. **Purity of the hMutLα variants used in this study.** Purified hMutLα variants (2 μg) were separated on a 12 % SDS polyacrylamide gel and stained with Coomassie Blue. Note that hMLH1 runs as a doublet on these gels, as previously reported (37). Due to the low expression levels, $L\alpha^{NA/NA}$ was obtained only in a partially-purified form in low amounts (approximately 0.5 μg loaded; see text for details). $L\alpha^{DN/WT}$ and $L\alpha^{DN/DN}$ could not be purified.

FIG. 5. **UV crosslinking of [α-^{32}P]-ATP to hMutLα variants.** Purified hMutLα variants (1 μg) were incubated with [α-^{32}P]-ATP. After short UV irradiation, the proteins were separated on a 7.5 % SDS polyacrylamide gel. *Left panel,* Gel stained with Coomassie Blue. *Right panel,* Autoradiograph of the same gel. A faint band was detected at the height of hPMS2 after very long exposures (data not shown).

FIG. 6. **ATP and magnesium binding induce a conformational change in the hMLH1 subunit. a,** 3 μg

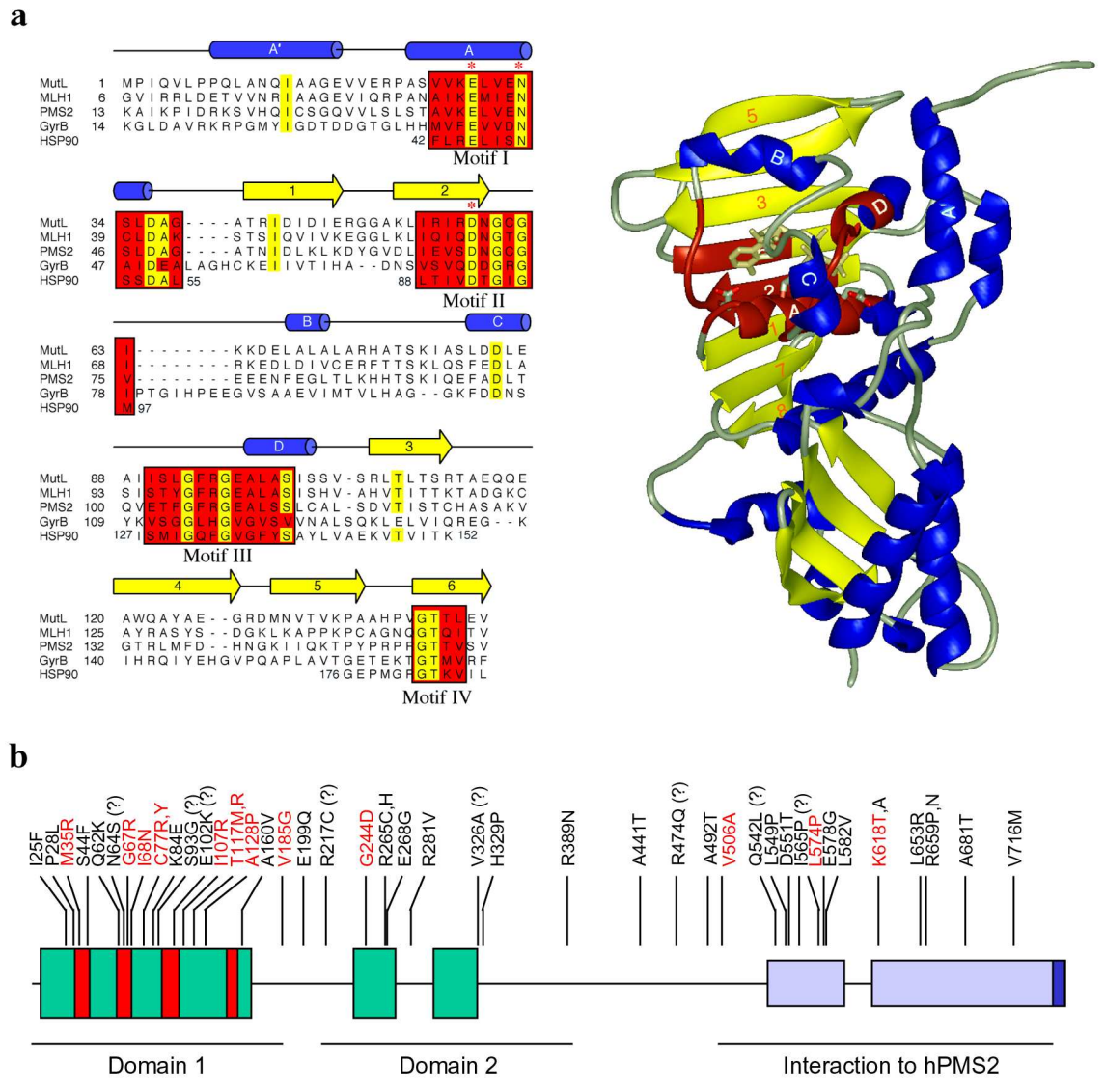
of purified wild type hMutLα were incubated with increasing concentrations of ATP and 1 mM Mg^{2+} for 15 minutes at 20 ÚC. The proteins were then partially digested with 10 ng trypsin for further 8 minutes at 20 ÚC and the tryptic peptides were separated on 10 % SDS polyacrylamide gels and visualized with Coomassie Blue. The proteolytic fragment appearing in the presence of >10 μM ATP is marked with an arrow. **b,** Appearance of the 39 kDa proteolytic fragment (arrow) is dependent on the presence of ATP and magnesium. The fragment was absent from the partial tryptic digest of $L\alpha^{NA/WT}$, which is unable to bind ATP in the active site of the hMLH1 subunit. This suggests that the fragment originates from the latter polypeptide (see text for details).

FIG. 7. **ATPase activity of hMutL**α. Purified $L\alpha^{WT/WT}$ or the catalytic single or double mutants $L\alpha^{EA/WT}$, $L\alpha^{WT/EA}$ and $L\alpha^{EA/EA}$ (4 μM) were incubated with 400 μM [γ-^{32}P]-ATP. After the indicated time, the samples were separated on 20 % denaturing polyacrylamide gels, exposed to Biomax MR film and the bands were quantitated using the ImageQuant software (Molecular Dynamics, Inc.). The assay was performed in triplicate using three independent preparations of the recombinant proteins (error bars show the standard deviation from the mean).

FIG. 8. **ATPase activity of hMutL**α **is required for mismatch correction.** Purified hMutLα variants (200 ng) were used to complement extracts of mismatch repair-deficient HCT116 cells. The substrates were M13 heteroduplexes carrying a G/T mispair and a strand discrimination signal (a nick) either 3 or 5 from the mismatch (see Experimental Procedures). Similar results were obtained when the HCT116 extracts were supplemented with extracts from Sf9 cells over-expressing the hMutLα variants, ruling out the possibility that the mismatch repair activity of the mutant heterodimers was lost during the purification procedure (data not shown). Because the repair activity of various MMR deficient cell lines fluctuates between 0 and 25% in this assay (data not shown),

levels below 25% are scored as MMR deficient

FIG. 9. **Interaction of hMutLα variants with hMutSα on heteroduplex DNA.** *Left panel,* Bandshift analysis performed with wild type hMutSα (75 nM) and hMutLα (150 nM), using ^{32}P-labeled 48-mer homoduplex G/C or a 48/49-mer heteroduplex oligonucleotide containing a one nucleotide IDL (4 nM). Where indicated, monoclonal antibodies against hMSH6 (1) or against hMLH1 (2) were added to the reactions. The free substrate was separated from protein-bound complexes on 4 % native polyacrylamide gels, which were then analyzed on a STORM PhosphoImager using the ImageQuant software (Molecular Dynamics, Inc.). Where indicated, reactions contained 2 mM $MgCl_2$ and/or 0.5 mM ATP. *Right panel,* Bandshift analysis carried out with hMutLα variants. All reactions contained wild type hMutSα (75 nM), the indicated hMutLα variants (150 nM) and 0.5 mM ATP. $MgCl_2$ was omitted from all reactions. Note that due to the low concentration of the purified protein, the reaction in lane 7 contained only 75 nM L$\alpha^{NA/NA}$. However, the presence of the band, albeit only a weak one, at the height of the supershift (**) suggests that also this mutant was able to interact with DNA-bound hMutSα.


a
A'
A
MutL 1 MPIQVLPPQLANQIAAGEVVERPASVVKELVEN
MLH1 6 GVIRRLDETVVNRIAAGEVIQRPANAIKEMIEN
PMS2 13 KAIKPIDRKSVHQICSGQVVLSLSTAVKELVEN
GyrB 14 KGLDAVRKRPGMYIGDTDDGTGLHHMVFEVVDN
HSP90 42 FLRELISN
Motif I
1
2
MutL 34 SLDAG----ATRIDIDIERGGAKLIRIRDNGCG
MLH1 39 CLDAK----STSIQVIVKEGGLKLIQIGDNGTG
PMS2 46 SLDAG----ATNIDLKLKDYGVDLIEVSDNGCG
GyrB 47 AIDEALAGHCKEIIVTIHA--DNSVSVCDLGRG
HSP90 55 88 LTIVDIGIG
Motif II
B
C
MutL 63 I--------KKDELALALARHATSKIASLDDLE
MLH1 68 I--------RKEDLDIVCERFTTSKLQSFEDLA
PMS2 75 V--------EEENFEGLTLKHHTSKIQEFADLT
GyrB 78 IPTGIHPEEGVSAAEVIMTVLHAG--GKFDDNS
HSP90 M 97
D
3
MutL 88 AISLGFRGEALASISSV-SRLTLTSRTAEQQE
MLH1 93 SISTYGFRGEALASISHV-AHVTITTKTADGKC
PMS2 100 QVETFGFRGEALSSLCAL-SDVTISTCHASAKV
GyrB 109 YKVSGGLHGVGVSVVNALSQKLELVIQREG--K
HSP90 127 ISMIGQFGVGFYSAYLVAEKVTVITK 152
Motif III
4
5
6
MutL 120 AWQAYAE--GRDMNVTVKPAAHPVGTTLEV
MLH1 125 AYRASYS--DGKLKAPPKPCAGNQGTQITV
PMS2 132 GTRLMFD--HNGKIIQKTPYPRPRGTTVSV
GyrB 140 IHRQIYEHGVPQAPLAVTGETEKTGTMVRF
HSP90 176 GEPMGRGTKVIL
Motif IV
b
I25F
P28L
M35R
S44F
Q62K
N64S (?)
G67R
I68N
C77R,Y
K84E
S93G (?)
E102K (?)
I107R
T117M,R
A128P
A160V
V185G
E199Q
R217C (?)
G244D
R265C,H
E268G
R281V
V326A (?)
H329P
R389N
A441T
R474Q (?)
A492T
V506A
Q542L (?)
L549P
D551T
I565P (?)
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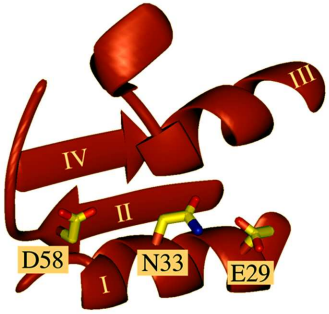
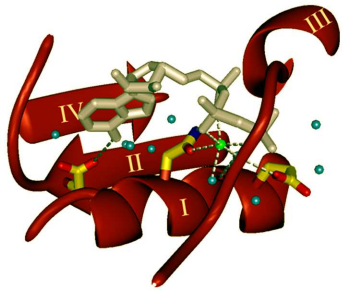
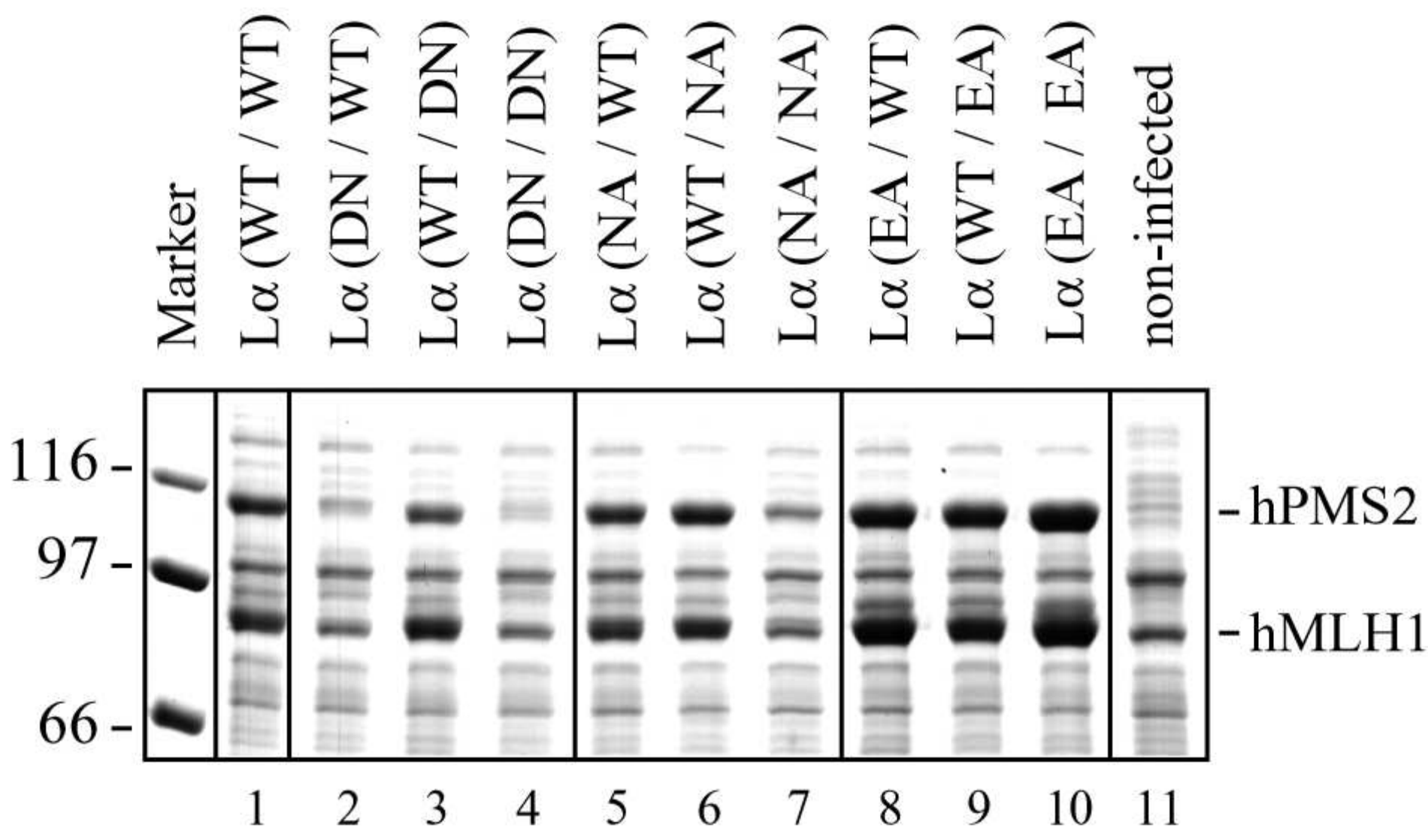
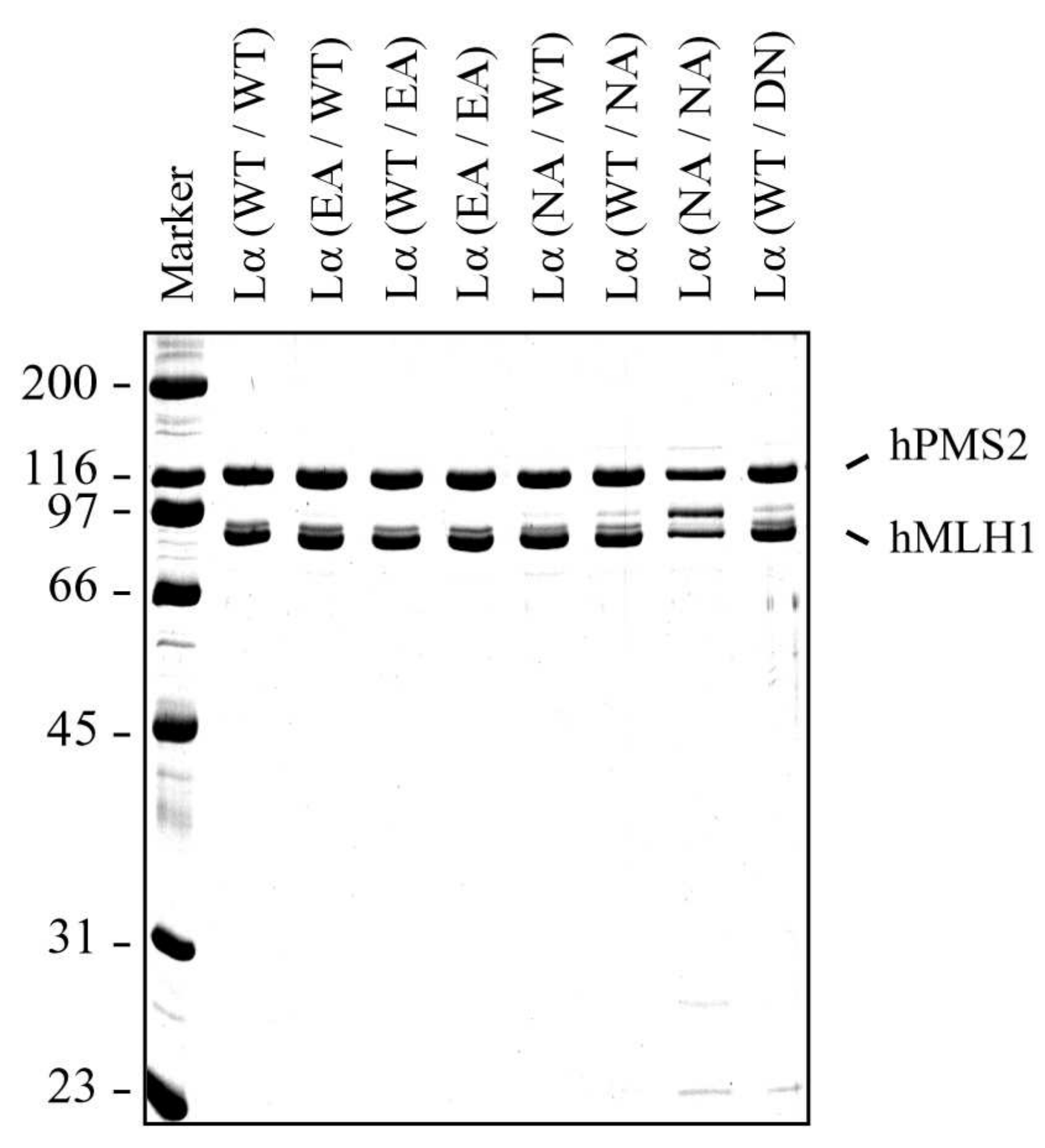
V716M
Domain 1
Domain 2
Interaction to hPMS2

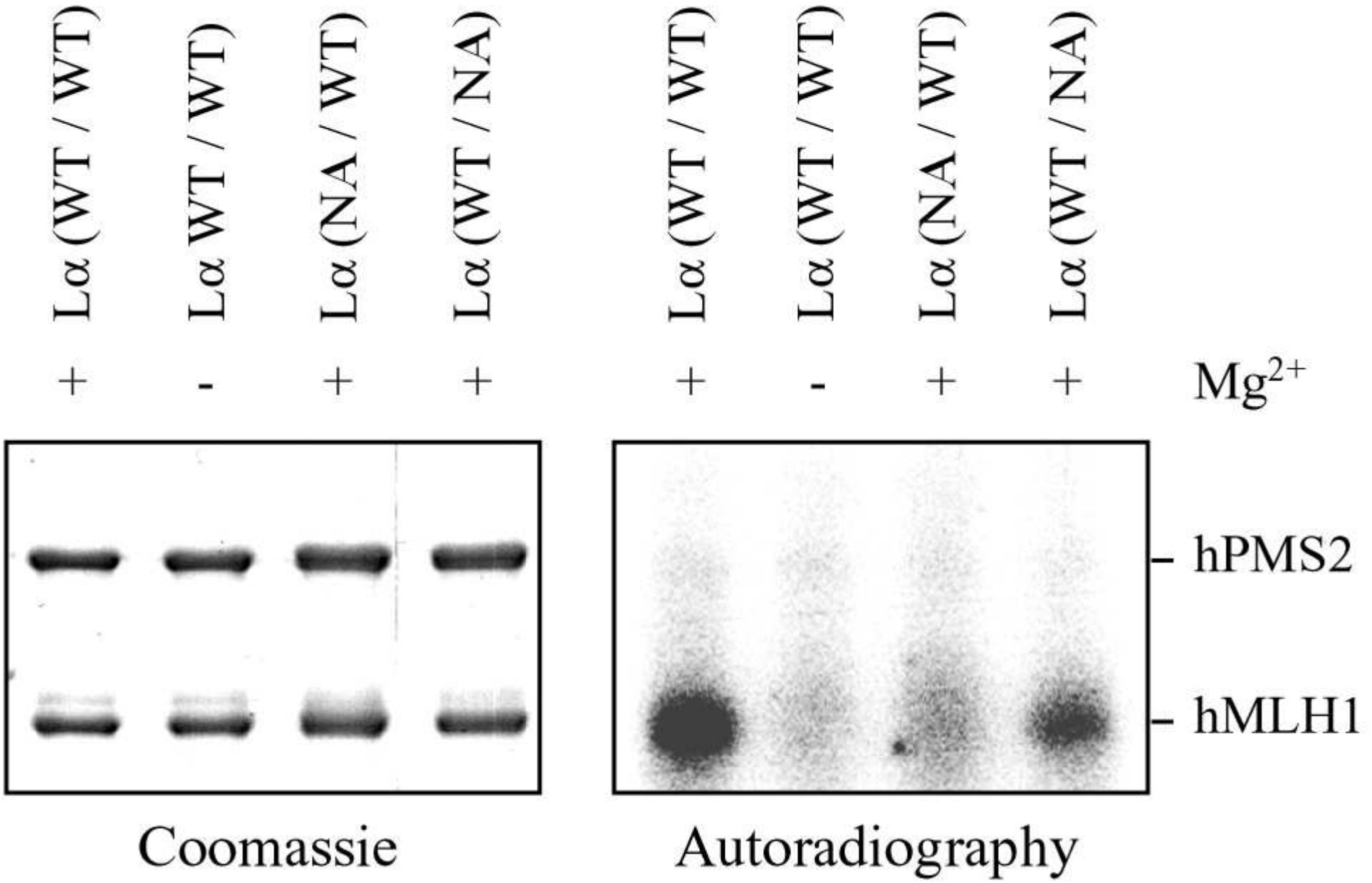


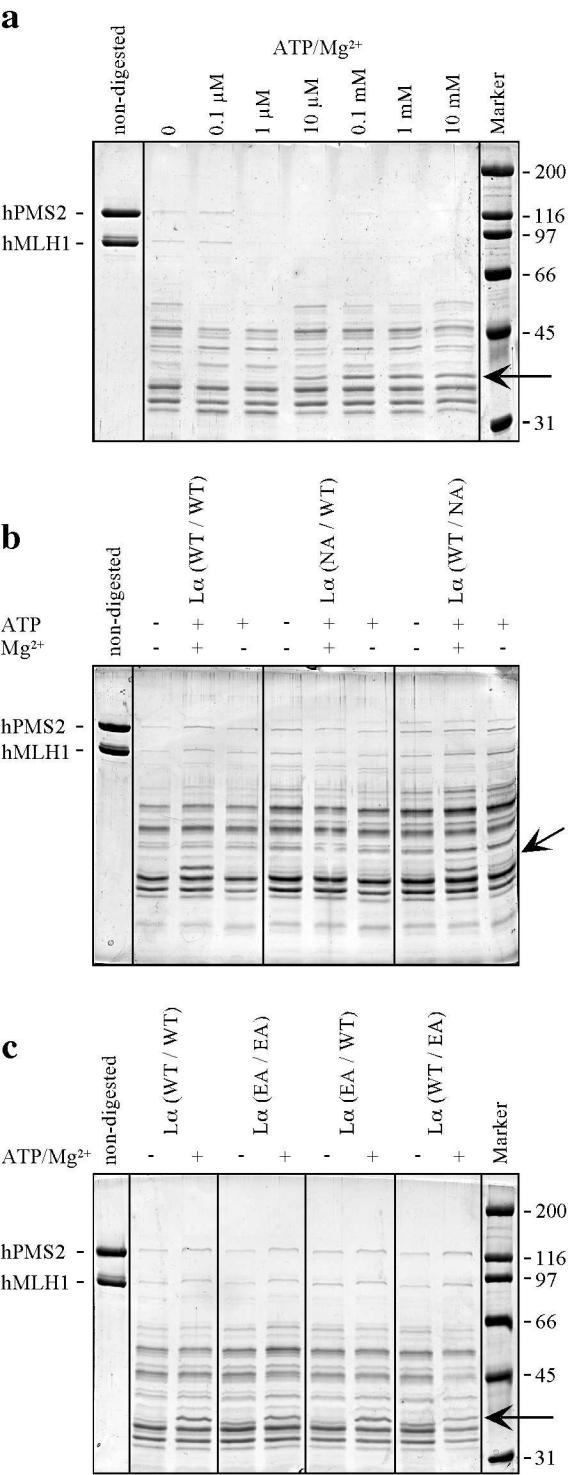


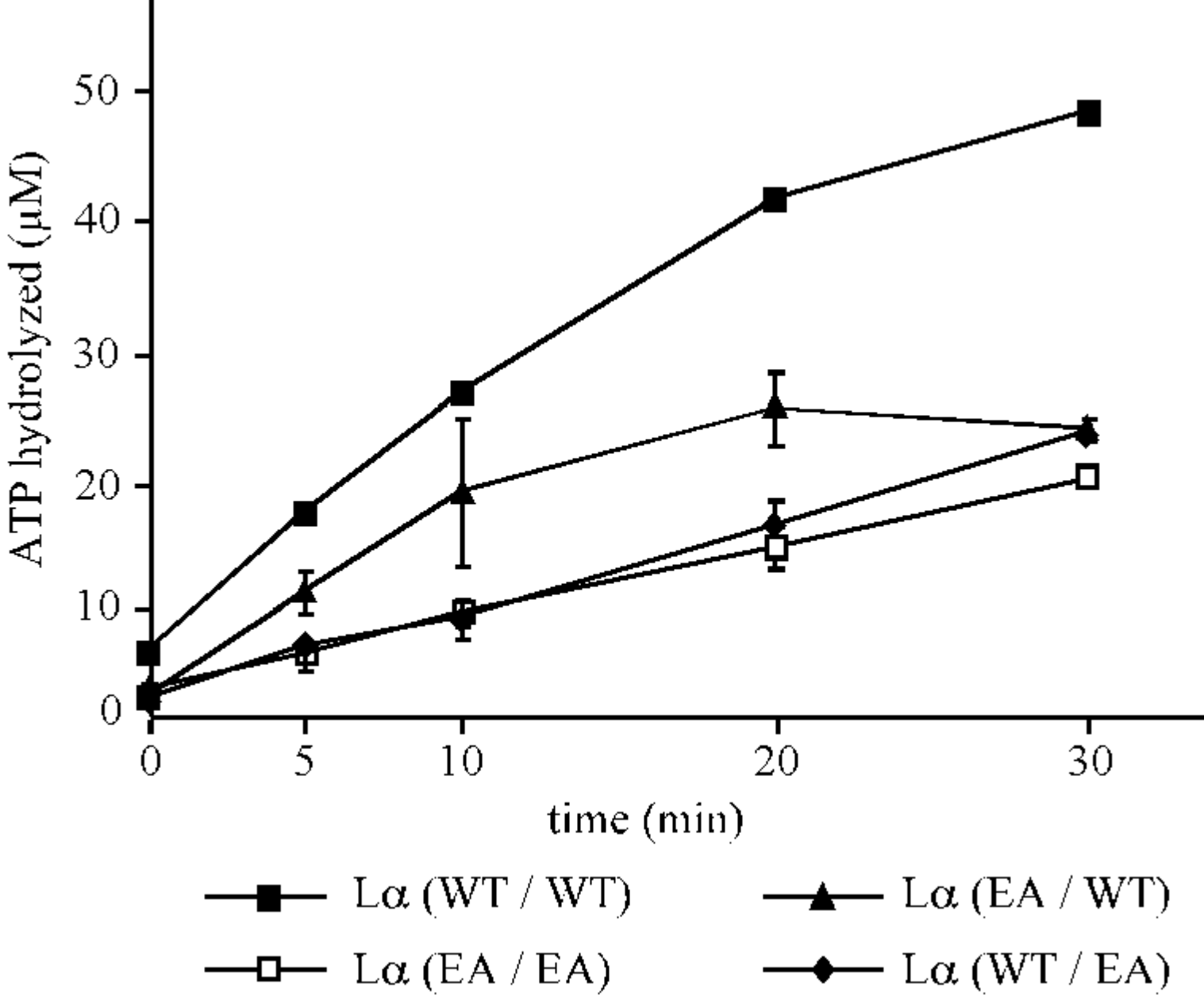
MutL	hMLH1	hPMS2	Function
E29	E34A	E41A	Catalytic Residue
N33	N38A	N45A	Binding to Mg^{2+}
D58	D63N	D70N	Binding to N6 of Adenine

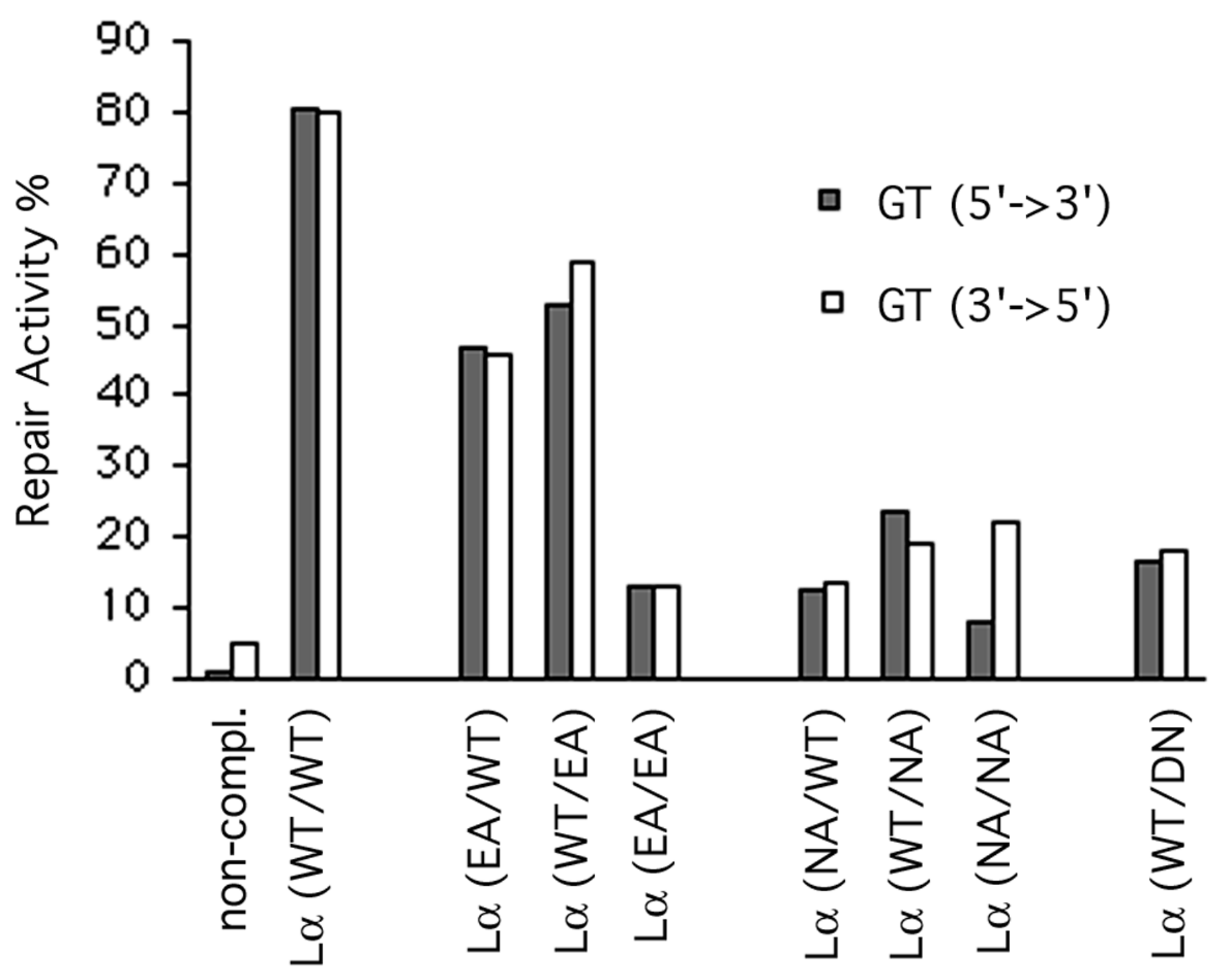

Marker
Lα (WT / WT)
Lα (DN / WT)
Lα (WT / DN)
Lα (DN / DN)
Lα (NA / WT)
Lα (WT / NA)
Lα (NA / NA)
Lα (EA / WT)
Lα (WT / EA)
Lα (EA / EA)
non-infected
116 -
97 -
66 -
- hPMS2
- hMLH1
1
2
3
4
5
6
7
8
9
10
11

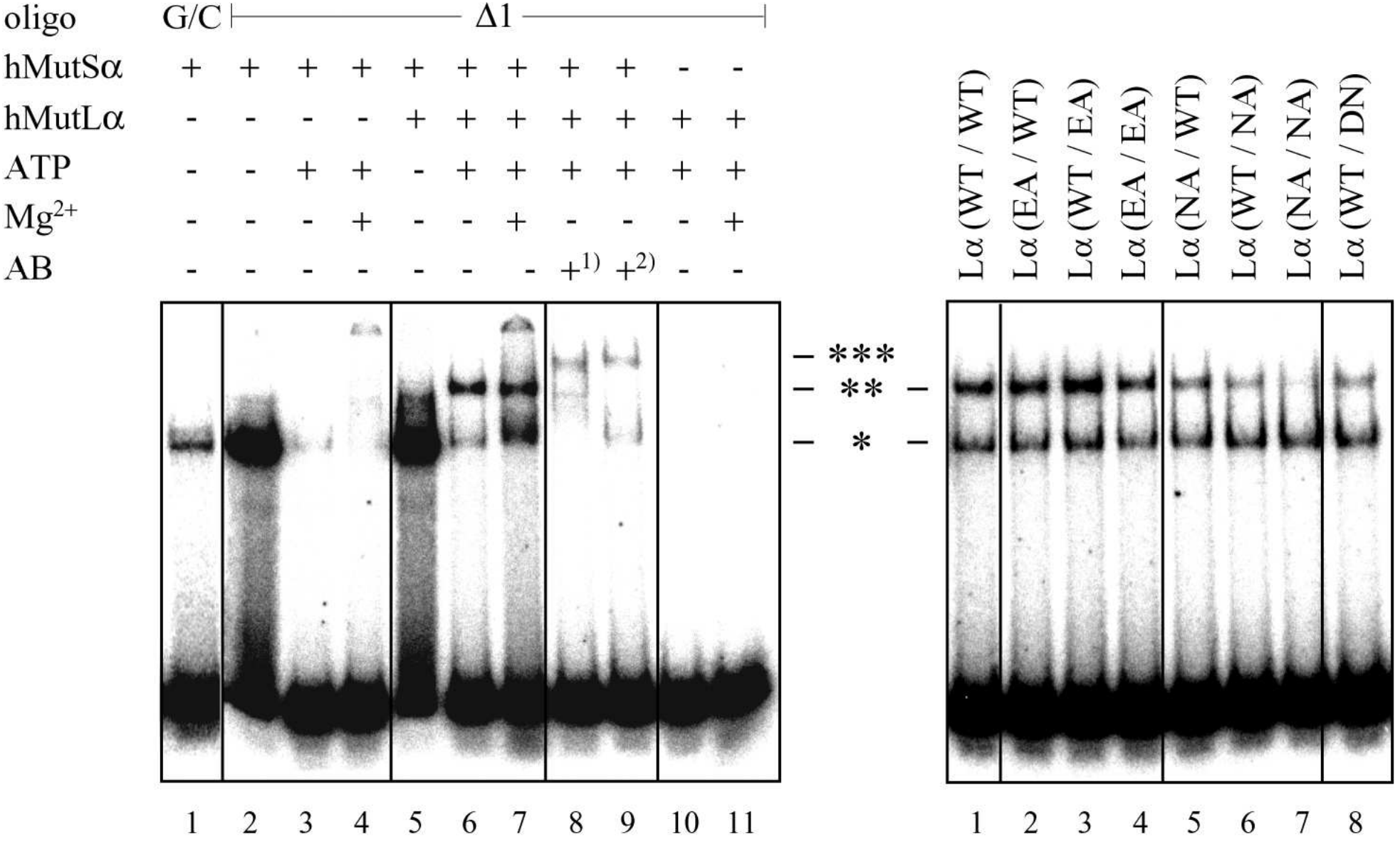

Marker
Lα (WT / WT)
Lα (EA / WT)
Lα (WT / EA)
Lα (EA / EA)
Lα (NA / WT)
Lα (WT / NA)
Lα (NA / NA)
Lα (WT / DN)
200
116
97
66
45
31
23
hPMS2
hMLH1


Lα (WT / WT)
Lα WT / WT)
Lα (NA / WT)
Lα (WT / NA)
+
-
+
+
Mg2+
hPMS2
hMLH1
Coomassie
Autoradiography


a
non-digested
ATP/Mg2+
0
0.1 µM
1 µM
10 µM
0.1 mM
1 mM
10 mM
Marker
hPMS2
hMLH1
200
116
97
66
45
31
b
non-digested
Lα (WT / WT)
Lα (NA / WT)
Lα (WT / NA)
ATP
Mg2+
hPMS2
hMLH1
c
non-digested
Lα (WT / WT)
Lα (EA / EA)
Lα (EA / WT)
Lα (WT / EA)
Marker
ATP/Mg2+
hPMS2
hMLH1
200
116
97
66
45
31


ATP hydrolyzed (μM)
50
40
30
20
10
0
0
5
10
20
30
time (min)
Lα (WT / WT)
Lα (EA / WT)
Lα (EA / EA)
Lα (WT / EA)


Repair Activity %
90
80
70
60
50
40
30
20
10
0
GT (5'->3')
GT (3'->5')
non-compl.
Lα (WT/WT)
Lα (EA/WT)
Lα (WT/EA)
Lα (EA/EA)
Lα (NA/WT)
Lα (WT/NA)
Lα (NA/NA)
Lα (WT/DN)


oligo G/C Δ1
hMutSα + + + + + + + + + - -
hMutLα - - - - + + + + + + +
ATP - - + + - + + + + + +
Mg2+ - - - + - - + - - - +
AB - - - - - - - +1) +2) - -
1 2 3 4 5 6 7 8 9 10 11
***
**
*
Lα (WT / WT)
Lα (EA / WT)
Lα (WT / EA)
Lα (EA / EA)
Lα (NA / WT)
Lα (WT / NA)
Lα (NA / NA)
Lα (WT / DN)
1 2 3 4 5 6 7 8